IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benvenisty	Atty Docket:	1822/113
Serial No.:	09/918,702	Art Unit:	1632
Date Filed:	July 31, 2001	Examiner:	Crouch, D.
Invention:	Directed Differentiation of Embryonic Stem cells		

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CERTIFICATE OF MAILING

I hereby certify that this document, along with any other papers referred to as being attached or enclosed, is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on September 21, 2004.

Barbara J. Carter, Ph.D.

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF BENVENISTY, M.D., PH.D. IN SUPPORT OF APPLICANT'S RESPONSE [37 C.F.R. § 1.132]

Dear Sir:

In support of the accompanying response to the Office Action mailed June 1, 2004 in the above-reference matter, I hereby declare as follows:

1. My name is Nissim Benvenisty, M.D., Ph.D. I am a Professor at The Hebrew University in Jerusalem, Israel, in the Department of Genetics. I was formerly the Vice Chair of the Institute of Life Sciences at The Hebrew University, and have been a visiting Professor in the Department of Genetics at Harvard University in Boston, MA, among

other positions. I have been awarded numerous prizes and fellowships, including the Teva Prize for excellent research in stem cells in 2003, the Herbert Cohn Chair in Cancer Research during 1999, the Rom Prize in Genetics in 1998, a Howard Hughes Postdoctoral Fellowship from 1991-1993, and a Fulbright Postdoctoral Fellowship from 1990-1991. I have published extensively in the field of stem cell research, and other areas, with over 65 publications, to date. I am also an inventor or co-inventor of a substantial number of patents involving (among other things) human embryonic stem cell research, and I am the sole inventor of the invention claimed in the current application. My further credentials are set forth in my Curriculum Vitae, which is attached hereto as Exhibit A.

2. I have read the action of June 1, 2004. This declaration is provided to clarify the record concerning human embryonic stem cell differentiation and research relative to mouse embryonic stem cell differentiation and research, and in particular the reasons why having the ability to direct differentiation in mouse ES cells does not translate into the ability to direct differentiation in human ES cells and does not render that important milestone obvious. This declaration is also provided to distinguish, on a technical level, why the prior art references cited in the Office Action, all dealing with mouse ES cell culture and differentiation, are not proper prior art references for the presently claimed invention, which deals with directed differentiation of human ES cells.

Consideration of the Prior Art

3. The Examiner rejected claim 10 because "the specification does not reasonably provide enablement for culturing embryonic cells without an extracellular matrix."

However, claim 10, which depends from claim 8, is fully enabled because although Example 1 describes culturing of embryoid bodies (EB)-derived embryonic cells in the presence of fibronectin, persons of ordinary skill in the field would understand that other methods of culturing may be used. For example, I have successfully cultured said embryonic cells in the presence of gelatin instead. Gelatin is not extracellular matrix (ECM) and, therefore, embryonic stem cells may be cultured in the absence of ECM. Example 1, and the methodology of Thomson et al. is a preferred way of culturing embryonic cells derived from EBs, but those skilled in the art would understand that other ways are also possible.

4. The Examiner rejected claims 8, 9, 11, 12, 60 and 65 under 35 U.S.C. 103(a) as being unpatentable over Thomson et al. (1998) in view of Shamblott et al. (1998) and further in view of Yuen et al. (1998). I disagree with the basis for the Examiner's rejection. Although Thomson affirms that the human ES cell lines that were generated "maintained the potential to form derivatives of all three embryonic germ layers" (See Thomson p. 1146, 1st column, lines 30-32), this potential was evidenced in the form of teratomas generated in SCID mice injected with said cell lines (*id.*, p. 1147, Figure 4). This finding is immaterial when considering that the problem that we are trying to solve in our laboratory is the development of human stem cell-derived differentiated cell lines that can eventually be used as material for transplantation.

5. Thomson showed the ability to generate teratomas, which is a form of tumor. Thomson's results do not point to the generation of differentiated cell lines of the three germ cell layers but rather, to the generation of tumor cells that display *markers* of the three *embryonic* germ layers, which is what characterizes these tumor cells. These are

not directly differentiated embryonic cells to a specific cell type, but rather embryonic stem cell-derived cell lines that form a tumor, and clearly would not be suitable for transplantation.

6. Furthermore, the only two molecular markers evaluated in Thomson's cell lines were human chorionic gonadotropin (HCG) and alpha-fetoprotein (AFP), which are also markers for *extra*-embryonic cells. HCG is expressed in trophoectoderm, while AFP is expressed in the yolk sac, both part of the extra-embryonic tissues. Therefore, besides the formation of teratomas, Thomson did not unequivocally prove the generation of differentiated cell lines of the three different germ layers.

7. Shamblott teaches the production of human EBs from human embryonic germ (hEG) cells. Human EG cells are very different from hES cells, with respect to the following:

	hEG cells	**hES cells**
-Origin	Primordial germ cells	Inner Cell Mass
-Imprinting status	Defective	Functional
-Growth potential	Maximum 20 passages	More than 100 passages

8. Clearly, any cell line deriving from hEG cells will be dissimilar from cell lines derived from hES cells. It is a well known fact to those skilled in the art that ES cells have an inherent pluripotency that is not reproducible by any other cell line, even if some cell line might show some pluripotent property, like for example in Shamblott's, where the hEG cells were able to form embryoid body-like structures. Nonetheless, it is still not correct to say that the embryoid body-like structures of Shamblott et al. are equivalent to

the embryoid bodies derived from hES cells. So, albeit these hEG cells formed EB-like structures, and differentiated into all three germ layers, it cannot be assumed that the Shamblott et al. differentiated cells have the same potential as cells derived from hES cells.

9. For example, an essential characteristic of ES cells is that their genetic material is "naïve" with respect to their imprinting status, and it is able to receive the correct signals to become imprinted throughout development. In contrast, EG Cells are already imprinted, and therefore cannot be imprinted "*de novo*". Those skilled in the art can appreciate the downfalls of lacking such feature. Furthermore, Shamblott et al. show spontaneous differentiation of EG cells, and not directed differentiation, as claimed in the present invention. Lastly, the differentiated cells developed by Shamblott et al. were able to grow for only a maximum of 20 passages (because they are derived from primordial germ cells), and so cannot be considered useful substitutes for transplantation material.

10. Therefore at every critical level it is fundamentally incorrect to consider the Shamblott et al. differentiated cells as equivalent to the EB-derived differentiated ES cells of the present invention. Unlike the cells produced in our laboratory, the Shamblott et al. cells cannot be imprinted *de novo*, they spontaneously differentiate and so cannot be directed to differentiate when required, and they cannot survive after more than 20 passages.

11. Yuen et al. teach the generation of a primitive erythroid cell line from *mouse* ES cells, not human ES cells. Although Yuen et al. mention the formation of EBs, they do not show said EBs, and certainly do not show the presence of EB molecular markers. Moreover, the Yuen et al. EBs differentiate only into an erythroid cell line, and not into

cells from all three germ layers as shown by the present inventors. Most importantly, this study was done in mouse cells, and not in human ES cells. Scientists working in the field of stem cell research understand that it does not follow that just because a procedure is successful in mice ES cells that it will be successful in human ES cells.

12. At the date of the present invention, it was only known in the field of stem cell research that it was possible to obtain EBs from mouse ES cells. However, it was not at all known, neither obvious, nor trivial, to obtain EBs from human ES cells. Reubinoff [Reubinoff et al., (2000) *Nature Biotechnology*, Vol 18, pp. 399-404] described how differentiation of human ES cell *in vitro* did not lead to the formation of embryoid bodies (see p. 401, 1st column, lines 26-29, and 2nd column, lines 1-100). In addition, Thomson [Thomson et al., (1995) *Proc. Natl. Acad. Sci. USA*, Vol. **92**, pp. 7844-7848 – see Exhibit B] was unable to show the formation of embryoid bodies in Rhesus monkey cells – which are closer in the evolutionary scale to human cells than marmoset cells, from which only sporadically EBs were formed [Thomson et al., (1996) *Biol. Reprod.*, Vol. **55**, pp. 254-259 – see Exhibit C]. Thus, the state of the art at the time of the invention was essentially that the formation of embryoid bodies from human ES cells, especially based on the technology used for mouse ES cells, was not possible.

13. It is erroneous to propose that without undue experimentation, it was obvious to combine the teachings of Thomson et al. (2000), Shamblott et al.(1998) and Yuen et al.(1998) and arrive at the present invention. If it was obvious, especially when considering how important the generation of embryoid bodies from human ES cells is for science in general and particularly for medicine, many other laboratories would have published such findings concomitantly. But except for the work in our laboratory, no

other laboratory in the world was successful in developing the technology of generating embryoid bodies from human ES cells.

14. In fact, in a News Focus in Science magazine (Vogel, G., (2002) *Science*, Vol. **295**, pp. 1818-1820 – see Exhibit D) under a section entitled "Fundamental Firsts", my achievements of both embryoid bodies formation and directed differentiation of human ES cells are highlighted (see p. 1819, column 2, first full paragraph ("Their first project was to test whether human ES cells, like those from mice, can form ... embryoid bodies. Initial studies by Thomson's group suggested they didn't.... But by learning how to grow the cells suspended in liquid ... [they] found that they could."

15. Later in that same section, my laboratory is credited with other firsts, verifying that I was first to successfully produce human differentiated ES cells. See last line of column 2 through column 3, line 10 – "Benvenisty and company scored several other firsts. In October 2000 the team ... published the first paper on how growth factors ... prompt human ES cells to mature into different types.")

16. The Examiner rejected claims 8-12, 14-16, 48, 51 and 52 under 35 U.S.C. 103(a) as being unpatentable over Thomson et al. (1998) in view of Shamblott et al. (1998) and in view of Bain et al. (1998). As mentioned above, Thomson teaches the ability of a human ES cell line to form teratomas and also shows that these cells express HCG and AFP. There is no unequivocal proof that this cell line can be directed to differentiate into cell lines from all three germ layers.

17. Shamblott teaches the formation of embryoid body-like structures from human embryonic germ cells, which originate from primordial germ cells, and thus do not have the same characteristics and properties as human ES cells. Moreover, Shamblott shows

random spontaneous differentiation of embryoid body-derived cells into cells from the three germ layers, but no directed differentiation of cells.

18. Bain reports the treatment of mouse ES cell-derived embryoid bodies with retinoic acid (RA) and their differentiation into the neuronal lineage. The technology of developing embryoid bodies from mouse ES cells and directing their differentiation was known and established at the time of the present invention. However, the techniques and protocols that applied for mouse ES cells were not adequate for human ES cells. Therefore, the establishment of embryoid bodies from human ES cells may be considered a new and not obvious technology, since it was developed at a time when all the research reported that such technology was not possible (Reubinoff et al, 2000). In view of the above, there was no motivation, at the time of the invention, to combine the teachings of the cited publications and to arrive at the present invention, since the *status quo* was that embryoid bodies from human ES cells cannot be produced.

19. The Examiner further rejected claims 8, 11, 13, 48, 51 and 52, and claim 55, under 35 U.S.C. 103(a) as being unpatentable over Thomson et al. (1998) in view of Shamblott et al. (1998) and further in view of Bain et al. (1998) and Wobus (1988). As mentioned above, Thomson teaches the ability of a human ES cell line to form teratomas and also shows that these cells express HCG and AFP. There is no unequivocal proof that this cell line can be directed to differentiate into cell lines from al three germ layers.

20. Shamblott teaches the formation of embryoid body-like structures from human embryonic germ cells, which originate from primordial germ cells, and thus do not have the same characteristics and properties as human ES cells. Moreover, Shamblott shows

randon spontaneous differentiation of embryoid body-derived cells into the cells from the three germ layers, but no directed differentiation of cells.

21. Bain reports the treatment of mouse ES cell-derived embryoid bodies with retinoic acid (RA) and their differentiation into the neuronal lineage. The technology of developing embryoid bodies from mouse ES cells and directing their differentiation was known and established at the time of the present invention.

22. Wobus et al. report the effect of nerve growth factor (NGF) on the differentiation pattern of mouse ES and embryocarcinoma cells (EC) cells, and showed nerve cells differentiation. However, the techniques and protocols that applied for mouse ES cells were not adequate for human ES cells. Therefore, the establishment of embryoid bodies from human ES cells may be considered a new and not obvious technology, since it was development at a time when all the research reported that such technology was not possible (Reubinoff et al., 2000). In view of the above, there was no motivation, at the time of the invention, to combine the teachings of the cited publications and to arrive at the present invention, since the *status quo* was that embryoid bodies form human ES cells cannot be produced, and therefore, no cell line derived from human ES cells could have been generated.

23. The main issue at stake in the present invention is the generation of differentiated cells from embryoid bodies from *human ES cells*. At the time of the present invention, as stated by Reubinoff (2000), "manipulations used in our laboratory and elsewhere to facilitate embryoid body formation and multilineage differentiation of mouse ES cells induced the death of human ES cells" (Reubinoff et al. *Nature Biotech.*, Vol. 18, p. 401 2nd column, lines 7-10) and therefore, it was not obvious to produce differentiated cells

from human EB's. In fact, when I attended a conference in 2001 and presented my results showing human embryoid body formation from human ES cells and subsequent directed differentiation of the human embryonic cells, and many experts in the field simply did not believe me, including Reubinoff, questioning my results intensely.

24. With respect to the Examiner's discussion of the results obtained by Reubinoff based on high density versus low density (smaller clumps of cells) or single cells, the present application uses semi-confluent cultures, which to a person skilled in the art means high-density cultures. It should also be noted that it is unlikely that the reason why Reubinoff did not succeed in generating human ES cell-derived embryoid bodies was the number of cells used. The present invention also makes use of semi-confluent cultures which are essentially high density cultures. Reubinoff did not use single cells because it is not possible (or rather, to this day there are no reports) to generate EBs from single cells.

25. I hereby declare that all statements made herein are of my own knowledge and that all statements made on information and belief are true; and further that these statements are being made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United

States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Benvenisty

Benvenisty, M.D., Ph.D.

Dated: September 10, 2004

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Exhibits:

A - Curriculum Vitae of Nissim Benvenisty, M.D., Ph.D.

B - Thomson et al. (1995) *Proc. Natl. Acad. Sci. USA*, Vol. **92**, pp. 7844-7848.

C - Thomson et al. (1996) *Biol. Reprod.*, Vol. **55**, pp. 254-259.

D - Vogel, G. (2002) *Science*, Vol. **295**, pp. 1818-1820.

Exhibit A.

NISSIM BENVENISTY

CURRICULUM VITAE

Date and Place of Birth : 9.10.1958; Israel.

Marital Status: Married + 3.

Education :

1983	M.D.	Faculty of Medicine, Hebrew University.
1986	Ph.D.	Department of Developmental Biochemistry, Hadassah Medical School, Hebrew University.

Employment and Related Training :

2002-	Professor, Department of Genetics, The Hebrew University, Jerusalem, Israel.
2002-2003	Head of Biology Teaching and Vice Chair, Institute of Life Sciences The Hebrew University, Jerusalem, Israel
1999-2000	Visiting Professor, Department of Genetics, Harvard University, Boston, USA
1998-2002	Associate Professor, Department of Genetics, The Hebrew University, Jerusalem, Israel.
1993-1998	Senior Lecturer, Department of Genetics, The Hebrew University, Jerusalem, Israel.
1990-1993	Research Fellow, Department of Genetics, Harvard Medical School, Boston, USA. Under supervision of Professor Philip Leder.
1986-1990	Israeli Army Medical Service.
1983-1986	Graduate Student, Department of Developmental Biochemistry, The Hebrew University, Jerusalem, Israel.
1985	Research Associate, Case Western Reserve University, Cleveland, USA.

1983-1984	Internship, Hadassah Hospital, Jerusalem, Israel.
1982-1985	Teaching biochemistry and molecular biology to medical students at The Hebrew University.

Awards and Fellowships :

1981	Awarded the Faculty Prize.
1982	Awarded a Fellowship at the Mount Sinai Hospital, New York - Program for outstanding students.
1982-1985	Foulkes Foundation Fellowship.
1985	Best Teacher Award for teaching biochemistry and molecular biology.
1988	Awarded the Senta Foulkes Prize (London)..
1990-1991	Awarded the Weizmann Postdoctoral Fellowship.
1990-1991	Awarded the Fulbright Postdoctoral Fellowship.
1991-1993	Awarded the Howard Hughes Postdoctoral Fellowship.
1993-1996	Awarded the Alon Fellowship.
1994	The Joseph H. and Belle R. Braun Senior Lectureship in Life Sciences.
1995-1998	Awarded Best Teacher in Genetics.
1995	Awarded the Hebrew University Prize for Young Scientist.
1996	Awarded the Naftali Prize.
1997	Awarded the Hestrin Prize in Biochemistry and Molecular Biology.
1998	Awarded the Rom Prize in Genetics
1999	The Herbert Cohn Chair in Cancer Research
1999-2000	Awarded the Yamagiwa-Yoshida Memorial International Cancer Study Fellowship.
2003	Awarded the Teva Prize for excellent research in stem cells

List of publications

1. **Benvenisty, N.**, Ben-Simchon, E., Cohen, H. Mencher, D., Meyuhas, O. and Reshef, L. : Control of the activity of phosphoenolpyruvate carboxykinase and the level of its mRNA in livers of newborn rats. Eur. J. Biochem. 132: 663-668 (1983).

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4. **Benvenisty, N.**, Mencher, D., Meyuhas, O., Razin, A. and Reshef, L. : Sequential changes in the methylation patterns of the rat phospho-enolpyruvate carboxykinase gene during development. Proc. Natl. Acad.Sci. USA 82: 267-271 (1985).

5. **Benvenisty, N.**, Szyf, M., Mencher, D., Razin, A. and Reshef, L. : Tissue-Specific hypomethylation and expression of rat phosphoenolpyruvate carboxykinase gene induced by in-vivo treatment of fetuses and neonates with 5-azacytidine. Biochemistry 24: 5015-5019 (1985).

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Sequential changes in the methylation patterns of the rat phospho-enolpyruvate carboxykinase gen during development. Gent. Abstr. 17: 115 (1985).

9. **Benvenisty, N.** and Reshef, L. : Direct introduction of genes into rats and the expression of the genes. Proc. Natl. Acad. Sci. USA 83: 9551-9555
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10. Nechushtan, H., **Benvenisty, N.**, Brandes, R. and Reshef, L. : Glucocorticoids control phosphoenolpyruvate carboxykinase gene expression in a tissue-specific manner. Nucl. Acids Res. 15: 6405-6417 (1987).

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15. **Benvenisty, N.** : A molecular view of tissue differentiation and development. J. Royal Coll. Phys. London 23: 156-160 (1989).

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23. **Benvenisty, N.** , Leder, A., Kuo. A., and Leder, P.: An embryonically expressed gene is a target for c-Myc regulation via the c-Myc binding sequence. Genes & Development 6: 2513-2523 (1992).

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30. Cohen, H., Trus, M., **Benvenisty, N.** and Reshef, L.: Characterization of expression of several hepatoma specific genes during liver development and upon premature induction by hormones. Eur. J. Biochem. 242: 550-556 (1996).

31. Ben-Porath, I. and **Benvenisty, N.**: Characterization of a tumor associated gene, a member of a novel family of genes encoding membrane glycoproteins. Gene 183: 69-75 (1996).

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39. Eden, A. and **Benvenisty, N.**: Involvement of branched-chain amino

acid amino transferase, a target for c-Myc activity, in apoptosis. FEBS Lett. 457: 255-261 (1999).

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46. Schuldiner, M., Eiges, R., Eden, A. Yanuka, O., Itskovitz-Eldor, J., Goldstein, R. and **Benvenisty, N.** (PI): Induced neuronal differentiation of human embryonic stem cells. Brain Research 913:201-205 (2001) (Cover).

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to identify c-MYC targets. Gene 292:91-99 (2002).

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Exhibit B.

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Developmental Biology

Isolation of a primate embryonic stem cell line

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ABSTRACT **Embryonic stem cells have the ability to remain undifferentiated and proliferate indefinitely *in vitro* while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. Here we report the derivation of a cloned cell line (R278.5) from a rhesus monkey blastocyst that remains undifferentiated in continuous passage for >1 year, maintains a normal XY karyotype, and expresses the cell surface markers (alkaline phosphatase, stage-specific embryonic antigen 3, stage-specific embryonic antigen 4, TRA-1-60, and TRA-1-81) that are characteristic of human embryonal carcinoma cells. R278.5 cells remain undifferentiated when grown on mouse embryonic fibroblast feeder layers but differentiate or die in the absence of fibroblasts, despite the presence of recombinant human leukemia inhibitory factor. R278.5 cells allowed to differentiate *in vitro* secrete bioactive chorionic gonadotropin into the medium, express chorionic gonadotropin α- and β-subunit mRNAs, and express α-fetoprotein mRNA, indicating trophoblast and endoderm differentiation. When injected into severe combined immunodeficient mice, R278.5 cells consistently differentiate into derivatives of all three embryonic germ layers. These results define R278.5 cells as an embryonic stem cell line, to our knowledge, the first to be derived from any primate species.**

Embryonic stem (ES) cells, derived from preimplantation embryos (1, 2), and embryonic germ (EG) cells, derived from fetal germ cells (3, 4), are undifferentiated, immortal cells capable of differentiating into derivatives of all three embryonic germ layers. Well-characterized ES and EG cells have been derived only from rodents (1, 2, 5, 6). Pluripotent cell lines have been derived from preimplantation embryos of several non-rodent species (7–10), but the developmental potentials of these cell lines remain poorly characterized. Mouse ES cells remain undifferentiated through serial passages when cultured in the presence of leukemia inhibitory factor (LIF) and differentiate in the absence of LIF (11). Mouse ES cells injected into syngeneic mice form teratocarcinomas that exhibit disorganized differentiation, with representatives of all three embryonic germ layers. Mouse ES cells combined with normal preimplantation embryos as chimeras and returned to the uterus participate in normal development (12). Because mouse ES cells can contribute to functional germ cells in chimeras, specific genetic changes can be introduced into the mouse germ line through the use of ES cell chimeras (13).

The mechanisms controlling differentiation of specific lineages can be studied with mouse ES cells grown *in vitro*; however, significant differences between early human and mouse development suggest that human development will be more accurately represented by primate ES cells. For example, human and mouse embryos differ in the timing of embryonic genome expression (14), in the structure and function of the fetal membranes and placenta (15), and in formation of an embryonic disc instead of an egg cylinder. Human embryonal carcinoma (EC) cells, which are pluripotent, immortal stem cells from teratocarcinomas, provide an important *in vitro* model for understanding human differentiation (16). Some EC cell lines can be induced to differentiate in culture (17), which results in the loss of specific cell surface markers [stage-specific embryonic antigen 3 (SSEA-3), SSEA-4, TRA-1-60, and TRA-1-81] and the appearance of new markers (16). When pluripotent human EC cells are injected into immunocompromised mice, they form teratocarcinomas, some with derivatives of all three embryonic germ layers. However, there are limitations to the use of human EC cells in the study of development. (*i*) The range of differentiation obtained from human EC cell lines is more limited than that obtained from mouse ES cells and varies widely between cell lines (18). (*ii*) All pluripotent human EC cell lines derived to date are aneuploid (19), suggesting EC cells may not provide a completely accurate representation of normal differentiation. (*iii*) Ethical considerations severely restrict the study of human embryos, often making it impossible to verify that *in vitro* results have significance in the intact embryo. None of these limitations would be present with nonhuman primate ES cell lines.

Here we report the isolation of an ES cell line (R278.5) from a rhesus monkey blastocyst. This cloned cell line remains undifferentiated and continues to proliferate for >1 year in culture, maintains a normal XY karyotype, and maintains the potential to differentiate into trophoblast and to derivatives of embryonic endoderm, mesoderm, and ectoderm. The morphology, cell surface markers, and growth factor requirements of these cells differ significantly from mouse ES cells but closely resemble human EC cells.

MATERIALS AND METHODS

Cell Line Isolation. Six days after ovulation, an azonal blastocyst was recovered by a nonsurgical uterine flush technique from a 15-year-old rhesus monkey (20). The trophectoderm was removed by immunosurgery (21) using a rabbit anti-rhesus spleen cell antiserum followed by exposure to guinea pig complement. The intact inner cell mass (ICM) was separated from lysed trophectoderm cells and plated on mouse embryonic fibroblasts [previously exposed to 3000 rads (1 rad = 0.01 Gy) γ-radiation] in medium consisting of 80% Dulbecco's modified Eagle medium (4500 mg of glucose per liter, with L-glutamine, without sodium pyruvate; GIBCO) with 20% fetal bovine serum (HyClone), 0.1 mM 2-mercaptoethanol (Sigma), 1% nonessential amino acid stock (GIBCO) (22), and 1000 units of cloned human LIF per ml (GIBCO). After 16 days of culture, a central mass of cells was removed from

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Abbreviations: CG, chorionic gonadotropin; ES, embryonic stem; EC, embryonal carcinoma; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, inner cell mass; LIF, leukemia inhibitory factor; RT-PCR, reverse transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SSEA, stage-specific embryonic antigen.

epithelial outgrowths, exposed for 3 min to 0.05% trypsin-EDTA (GIBCO), gently dissociated by pipetting through a micropipette, and replated on mouse embryonic fibroblasts. After 3 weeks of growth, colonies with a morphology resembling human EC cells were selected and expanded. At five passages, individual cells were selected by micropipette and plated in individual wells of a 96-well plate (Falcon) with mouse embryonic fibroblast feeder layers. One clone with a normal karyotype (R278.5) was expanded for further analysis.

Cell Surface Markers. R278.5 cells grown on a layer of mouse embryonic fibroblasts were used to examine the expression of cell surface markers. Alkaline phosphatase was detected histochemically following fixation of cells with 100% ethanol using "Vector red" (Vector Laboratories) as a substrate, as described by the manufacturer. The SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 antigens were detected by immunocytochemistry with specific primary monoclonal antibodies (gifts of Peter Andrews, University of Sheffield, U.K.) (16, 23–25) and localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC system, Vector Laboratories).

***In Vitro* Differentiation.** R278.5 cells were plated at low density (≈5000 cells/cm^2 of surface area) in the absence of fibroblasts on gelatin-treated four-well tissue culture plates (Nunc) in the same medium as that used for initial cell line isolation, but with 0–10^4 units of added human LIF per ml (GIBCO). The resulting differentiated cells were photographed 8 days after plating.

A mouse Leydig cell bioassay (26) was used to measure luteinizing hormone/chorionic gonadotropin (CG) activity in medium conditioned for 2 days either by undifferentiated R278.5 cells (at 80% confluence on fibroblast feeder layers) or by spontaneously differentiated R278.5 cells (cultured for 2 weeks after achieving confluence on fibroblast feeders). The relative levels of the mRNAs for α-fetoprotein and the α- and β-subunits of CG relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (27) using RNA from the same undifferentiated and differentiated cells. The PCR primers for human G3PDH (Clontech) do not amplify mouse G3PDH mRNA. Primers for human α-fetoprotein mRNA flank the seventh intron (5′ primer, 5′-GCTGGATTGTCTGCAGGATGGGGAA; 3′ primer, 5′-TCCCCTGAAGAAAATTGGTTAAAAT) and amplify a cDNA of 216 bp. Primers for the β-subunit of human CG flank the second intron (5′ primer, 5′-*ggatc*CACCGTCAACACCACCATCTGTGC; 3′ primer, 5′-*ggatc*CACAGGTCAAAGGGTGGTCCTTGGG) (nucleotides added to the CGβ sequence to facilitate subcloning are shown in italics) and amplify a cDNA of 262 bp. The primers for the CGα subunit were based on sequences of the first and fourth exon of the rhesus gene (28) (5′ primer, 5′-*gggaattc*GCAGTTACTGAGAACTCACAAG; 3′ primer, 5′-*gggaattc*GAAGCATGTCAAAGTGGTATGG) and amplify a cDNA of 556 bp. The identity of all cDNAs was verified by sequencing (not shown).

For RT-PCR, 1–5 μl of total R278.5 RNA was reverse transcribed, and 1–20 μl of reverse transcription reaction was subjected to the PCR in the presence of 2.5 μCi of deoxycytidine 5′-[α-^{32}P]triphosphate (1 Ci = 37 GBq; DuPont). The number of amplification rounds that produced linear increases in target cDNAs and the relation between input RNA and amount of PCR product were empirically determined. Following agarose gel electrophoresis, DNA bands of interest were cut out and radioactivity was determined by liquid scintillation spectroscopy. The ratio of cpm in a specific PCR product relative to cpm of G3PDH PCR product was used to estimate the relative levels of mRNAs among differentiated

Tumor Formation in Severe Combined Immunodeficient (SCID) Mice. In the passage immediately prior to SCID mouse injection (7 months after initial derivation of R278), karyotypes of R278.5 were confirmed as euploid. Approximately 5 × 10^5 R278.5 cells were injected either into the rear leg muscles (seven mice) or into the testis (two mice) of 8- to 12-week-old male SCID mice. The resulting tumors were fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8–15 weeks of development.

RESULTS

The morphology and cell surface markers of R278.5 cells (Fig. 1*A*) more closely resembled human EC cells than mouse ES cells. R278.5 cells had a high nucleus/cytoplasm ratio and prominent nucleoli, but rather than forming compact, piled-up colonies with indistinct cell borders similar to mouse ES cells, R278.5 cells formed flatter colonies with individual, distinct cells. R278.5 cells expressed alkaline phosphatase activity and the cell surface antigens SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 2), cell surface markers characteristic of human EC cell lines (16). Although cloned human LIF was present in the medium at cell line derivation and for initial passages, R278.5 cells grown on mouse embryonic fibroblasts without exogenous LIF remained undifferentiated and continued to proliferate. R278.5 cells plated on gelatin-treated tissue culture plates without fibroblasts differentiated to multiple cell types or failed to attach and died, regardless of the presence or absence of exogenously added human LIF (Fig. 1*B*).

The mRNA for α-fetoprotein, a marker for endoderm, increased substantially with *in vitro* differentiation (Fig. 3). α-Fetoprotein is expressed by extra-embryonic (yolk sac) and embryonic (fetal liver and intestines) endoderm. Epithelial cells resembling extraembryonic endoderm were present in cells differentiated *in vitro* from R278.5 cells (Fig. 1*B*).

Luteinizing hormone activity, an indication of CG secretion and trophoblast differentiation, was present in culture medium collected from differentiated cells [3.89 milli-international units (mIU)/ml] but not in medium collected from undiffer-

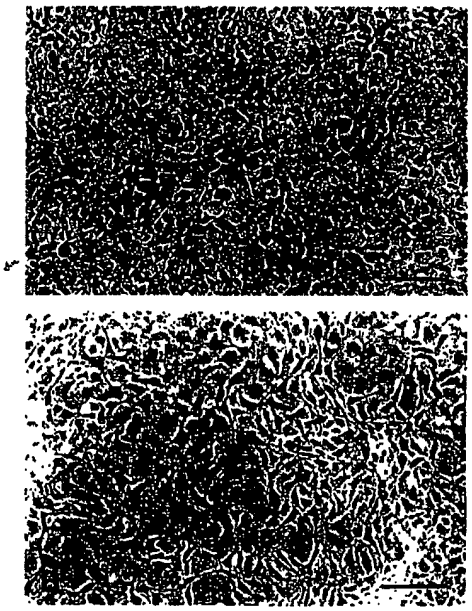

FIG. 1. Colony morphology and *in vitro* differentiation of cell line R278.5. (*A*) Undifferentiated R278.5 cells. Note the distinct cell borders, high nucleus to cytoplasm ratio, and prominent nucleoli. (Bar = 100 μm.) (*B*) Differentiated cells 8 days after plating R278.5 cells on gelatin-treated tissue culture plastic with 10^3 units of added human

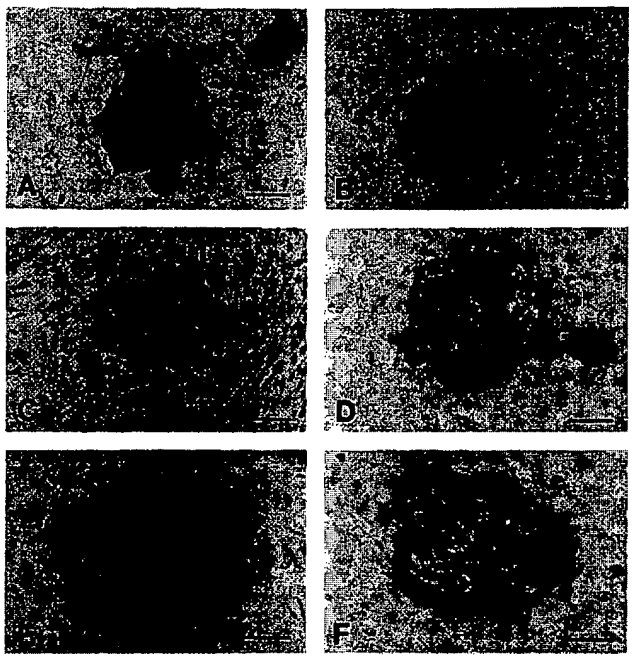

FIG. 2. Expression of cell surface markers by undifferentiated R278.5 cells. (*A*) Alkaline phosphatase. (*B*) SSEA-1. (*C*) SSEA-3. (*D*) SSEA-4. (*E*) TRA-1-60. (*F*) TRA-1-81. (Bars = 100 μm.) SSEA-3 staining of R278.5 cells was consistently weaker than the other positive antigens, and cell staining intensity varied within and between colonies.

entiated cells (<0.03 mIU/ml). The mRNAs for the CG subunits were readily detectable in the differentiated cells, although the relative level of the CGβ subunit mRNA was considerably lower than that for the CGα subunit (Fig. 4). The relative level of the CGα mRNA was quite low in undifferentiated cells, but the relative level was increased 23.9-fold after differentiation. The levels of the CGβ mRNA, on the other hand, increased only about 2-fold after differentiation for 2 weeks. Minor subpopulations of R278.5 cells differentiated even in the presence of fibroblasts, and the low level of α-fetoprotein, CGα, and CGβ mRNA present prior to the removal from fibroblasts could have been from these cells.

All SCID mice injected with R278.5 cells in either intramuscular or intratesticular sites formed tumors, and tumors in both sites demonstrated a similar range of differentiation. The

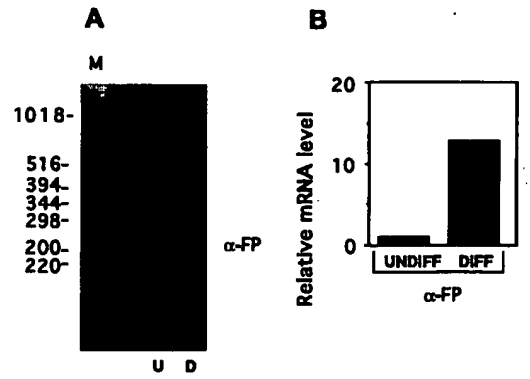

FIG. 3. Expression of α-fetoprotein mRNA. (*A*) PCR amplification of α-fetoprotein (αFP) cDNA from reverse-transcribed total RNA from undifferentiated (U) and differentiated (D) R278.5 cells. The DNA size markers (M) are indicated in bp. (*B*) The α-fetoprotein mRNA levels are expressed relative to the levels of the mRNA for G3PDH in each sample (not shown) as described in the text. Similar results were obtained in a second independent differentiation experiment.

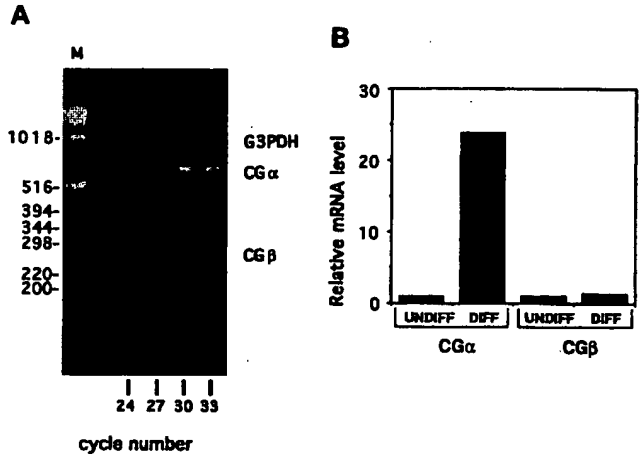

FIG. 4. Expression of CG subunit mRNA. (*A*) PCR amplification of cDNAs for G3PDH, CGα, and CGβ subunits from reverse-transcribed total RNA from differentiated R278.5 cells. The DNA size markers (M) are indicated in bp. (*B*) Relative levels of CGα and CGβ mRNAs in undifferentiated and differentiated R278.5 cells. Total RNA from cultured cells was analyzed for CG mRNA levels by RT-PCR and expressed relative to the levels of G3PDH mRNA. Similar results were obtained in a second independent differentiation experiment.

oldest tumors examined (15 weeks) had the most advanced differentiation, and all had abundant, unambiguous derivatives of all three embryonic germ layers, including ciliated columnar epithelium and nonciliated columnar epithelium (probable respiratory and gut epithelium; endoderm); bone, cartilage, smooth muscle, striated muscle (mesoderm); ganglia, other neural tissue, and stratified squamous epithelium (ectoderm), and other unidentified cell types (Fig. 5). Neural tissue included stratified cellular structures with remarkable resemblance to developing neural tube (Fig. 5*D*). Gut-like structures were often encircled by multiple layers of smooth muscle and were sometimes lined by villi with columnar epithelium interspersed with scattered mucus-secreting goblet cells (Fig. 5 *A* and *F*). Stratified squamous epithelium often contained well-differentiated hair follicles with hair shafts (Fig. 5*C*).

DISCUSSION

To our knowledge, there have been no previous reports of the isolation of a primate ES cell line. The characteristics that define R278.5 cells as ES cells include indefinite (>1 year) undifferentiated proliferation *in vitro*, maintenance of a normal karyotype, and potential to differentiate to derivatives of trophectoderm and all three embryonic germ layers. The development of complex structures in tumors in SCID mice with remarkable resemblance to normal hair follicles, neural tube, and gut demonstrates the ability of R278.5 cells to participate in complex developmental processes requiring coordinated interactions between multiple cell types. In the mouse embryo, the last cells capable of contributing to derivatives of trophectoderm and ICM are early ICM cells of the expanding blastocyst (29). The timing of commitment to ICM or trophectoderm has not been established for any primate species, but the potential of R278.5 cells to contribute to derivatives of both suggests that they most closely resemble early totipotent embryonic cells. The very limited ability of mouse ES cells to contribute to trophoblast in chimeras (30) suggests that the R278.5 cells represent an earlier developmental stage than mouse ES cells or that the ability of ICM cells to form trophectoderm persists longer in primates. Human EC cells share the ability of R278.5 cells to differentiate to trophoblast *in vitro* (16) and this potential may be a general distinguishing property of primate ES cell lines.

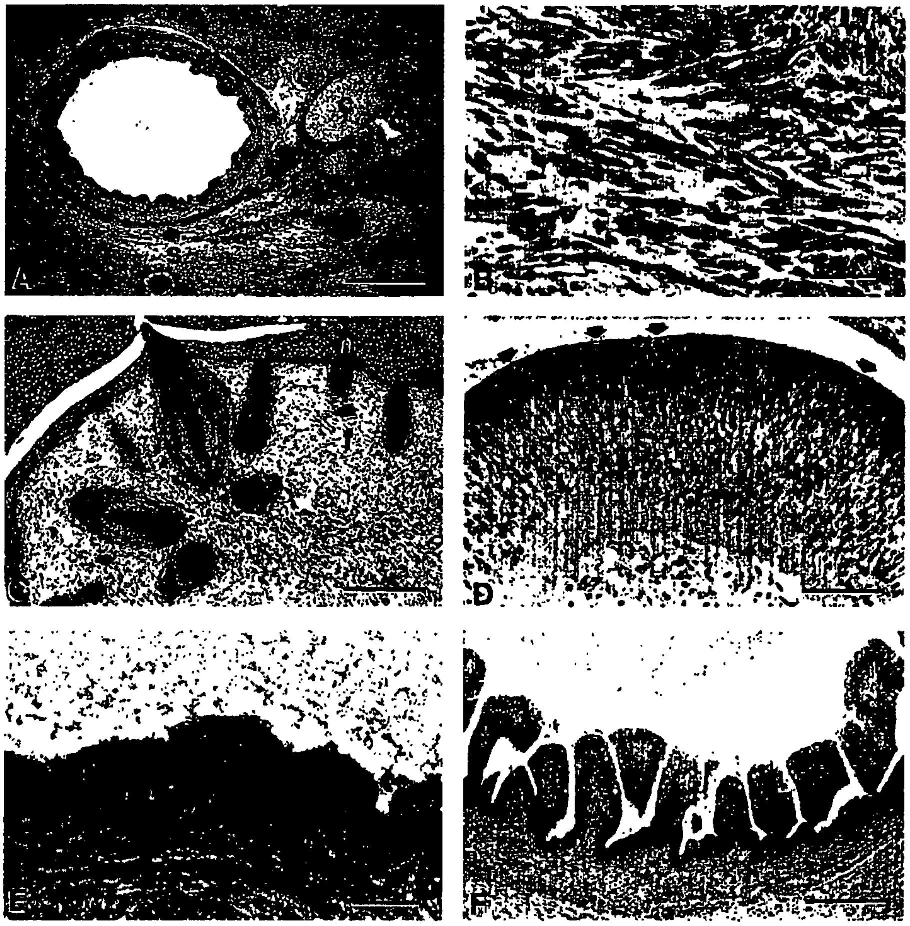

FIG. 5. Tumors formed by R278.5 cells injected into SCID mice and examined at 15 weeks. (*A*) Low-power field demonstrating disorganized differentiation of multiple cell types. A gut-like structure is encircled by smooth muscle (s), and elsewhere foci of cartilage (c) are present. (Bar = 400 μm.) (*B*) Striated muscle. (Bar = 40 μm.). (*C*) Stratified squamous epithelium with several hair follicles. The labeled hair follicle (f) has a visible hair shaft. (Bar = 200 μm.). (*D*) Stratified layers of neural cells in the pattern of a developing neural tube. An upper "ventricular" layer, containing numerous mitotic figures (arrows), overlies a lower "mantle" layer. (Bar = 100 μm.) (*E*) Ciliated columnar epithelium. (Bar = 40 μm.) (*F*) Villi covered with columnar epithelium with interspersed mucus-secreting goblet cells. (Bar = 200 μm.)

The only cells known to express the combination of markers alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 other than R278.5 cells are human EC cells (16, 25, 31). This expression pattern contrasts with undifferentiated mouse ES and EC cells, which instead express SSEA-1 and do not express SSEA-3, SSEA-4, TRA-1-60, or TRA-1-81 (23, 24). Differentiation of human EC cells such as NTERA2 cl.D1 (17) results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression and an increased SSEA-1 expression (16). These antigens have yet to be studied in early human or nonhuman primate embryos, and their functions are unknown, but their shared expression by R278.5 cells and human EC cells suggests a close embryological similarity.

In the absence of fibroblast feeder layers, soluble LIF fails to prevent the differentiation of R278.5 cells or of feeder-dependent human EC cells (19). The factors that fibroblasts produce that prevent the differentiation of R278.5 cells or feeder-dependent human EC cells are unknown. Other factors that fail to support the growth of feeder-dependent human EC cells in the absence of feeder layers include oncostatin M and ciliary neurotrophic factor (19), both of which can substitute for LIF in preventing the differentiation of mouse ES cells (32, 33). A trypsin-sensitive factor from a human yolk sac carcinoma cell line (GCT 44) supports the growth of feeder-dependent human EC cells in the absence of fibroblasts, but the factor has not yet been purified (19).

Although exogenous LIF was added during the initial derivation without added LIF. We have also recently derived two additional cell lines (R366 and R367) from four additional rhesus blastocysts, using the same techniques as described for R278.5 cells, but without added LIF (data not shown). R366 and R367 cells have normal karyotypes and continue to proliferate *in vitro* for at least 3 months. R366 and R367 cell lines have not yet been tested for tumor formation in SCID mice, but they are indistinguishable from R278.5 cells in undifferentiated morphology, growth characteristics, and *in vitro* differentiation in the absence of feeder layers.

The differentiation of R278.5 cells to trophoblast was demonstrated by the expression of CGα and CGβ subunit mRNAs and the secretion of bioactive CG into the culture medium by differentiated ES cells. We were surprised to note that while the relative levels of the CGα subunit were increased >20 times in differentiated cells, the relative levels of the CGβ subunit only changed about 2-fold. The fact that CG secretion increased substantially with differentiation may mean that under our *in vitro* culture conditions, expression of the CGα subunit is limiting for CG secretion. CGβ subunit mRNA is detectable in human preimplantation embryos as early as the eight-cell stage, which is before trophectoderm differentiation (34), consistent with a low level of CGβ mRNA expression in undifferentiated R278.5 cells. Although there may be some coordinate mechanisms regulating CGα and CGβ gene transcription in the placenta (35), it is clear that there is differential regulation of these genes *in vitro* and *in vivo* (36). Since the

and extravillous trophoblasts (37), further studies are needed to determine the phenotype of the trophoblasts derived from R278.5 cells.

Primate ES cells will be particularly useful for *in vitro* developmental studies of lineages that differ substantially between humans and mice. However, the most accurate *in vitro* model of the differentiation of human tissues would be provided by human ES cells. In one published report, ICM-derived cells from spare *in vitro* fertilized human embryos were cultured with LIF in the absence of feeder layers, and, although alkaline phosphatase positive cells proliferated, they failed to survive beyond two passages (38). These results suggest that soluble LIF alone will not prevent the differentiation of human ES cells, just as it fails to prevent the differentiation of rhesus ES cells. The growth of rhesus monkey ES cells in culture conditions that support feeder-dependent human EC cells suggests that similar conditions may support human ES cells.

Human ES cells would offer exciting new possibilities for transplantation medicine. Because ES cells have the developmental potential to give rise to all adult cell types, any disease resulting from the failure of specific cell types would be potentially treatable through the transplantation of differentiated cells derived from ES cells. Because ES cells are immortal cell lines, they could be genetically manipulated prior to differentiation either to reduce immunogenicity or to give them new properties to combat specific diseases. Rhesus monkey ES cells and rhesus monkeys will be invaluable for testing the safety and efficacy of the transplantation of specific cell types for the treatment of specific diseases. Because of the range of diseases potentially treatable by this approach, elucidating the basic mechanisms controlling the differentiation of primate ES cells has dramatic clinical significance.

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Exhibit C.

BIOLOGY OF REPRODUCTION 55, 254–259 (1996)

Pluripotent Cell Lines Derived from Common Marmoset (*Callithrix jacchus*) Blastocysts[1]

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ABSTRACT

We report the derivation of eight pluripotent cell lines from common marmoset (*Callithrix jacchus*) blastocysts. These cell lines are positive for a series of markers (alkaline phosphatase, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81) that characterize undifferentiated human embryonal carcinoma cells and rhesus embryonic stem cells. All eight cell lines had a modal chromosome number of 46; seven cell lines were XX and one was XY. Two cell lines (Cj11 and Cj62) were cultured continuously for over a year and remained undifferentiated and euploid. In the absence of fibroblast feeder layers, these cell lines differentiated to multiple cell types, even in the presence of leukemia inhibiting factor. Differentiated cells secreted bioactive CG into the culture medium and expressed α-CG, β-CG, and α -fetoprotein mRNA, indicating trophoblast and endoderm differentiation. Bioactive CG secretion in differentiating cells was increased substantially in the presence of GnRH agonist D-Trp[6]-Pro[9]-NHEt. When grown at high densities, these cells formed embryoid bodies with a close resemblance to early postimplantation embryos, including the formation of a yolk sac, amnion, and an embryonic disc with an early primitive streak. These results make these pluripotent cells strong candidates for marmoset embryonic stem cells.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cell lines capable of contributing to derivatives of all three embryonic germ layers even after prolonged culture [1–3]. Mouse ES cells in chimeras sometimes contribute to germ cells, thus providing a vehicle for introducing genetic changes into the germ line [4]. Because homologous recombination allows the alteration of specific loci of the genome, mouse ES cells allow the production of very specific models of human genetic diseases [5]. However, because of the differences between human and mouse development, anatomy, and physiology, transgenic mice can provide only a limited understanding of some human diseases. In addition, the testing of new therapies in transgenic mice is limited by mouse size, life span, and physiology. Transgenic primate models would increase our understanding of the pathogenesis of specific diseases and allow the testing of new therapies. In transgenic primates, therapeutic efficacy for treating degenerative neural diseases, such as Alzheimer's disease, could be assessed not only by morphological and biochemical changes in the brain, but by changes in complex behaviors.

We have recently reported the isolation of ES cells from the rhesus monkey that are immortal, have a stable normal karyotype, and have the potential to differentiate to derivatives of trophectoderm and all three embryonic germ layers [6]. Rhesus monkey ES cells provide a powerful new in vitro model for understanding the differentiation of human tissues, but the reproductive biology of rhesus monkeys makes testing the ability of these cells to contribute to the germ line in chimeras impractical. The rhesus monkey, which is an Old World primate species, has single young, reaches sexual maturity at 4–5 yr, and has an ovarian cycle that cannot be routinely synchronized. The common marmoset, a New World primate species, has more favorable reproductive characteristics for experimental primate embryology, including the natural birth of twins or triplets, an early age at sexual maturity (about 18 mo), and an ovarian cycle that can be synchronized with prostaglandins, thus allowing efficient embryo collection and transfer [7–9].

Here we report the derivation of eight pluripotent cell lines from common marmoset blastocysts that closely resemble rhesus ES cells and human embryonal carcinoma (EC) cells in morphology, growth characteristics, cell surface markers, and in vitro differentiation. Because of the reproductive characteristics of the common marmoset, it will be possible to define the developmental potential of these pluripotent cell lines in chimeras with normal embryos in vivo, initiating exciting advances in experimental primate embryology.

MATERIALS AND METHODS

Embryo Recovery and Cell Line Isolation

For embryo donors, female marmosets greater than 2 yr of age and demonstrating regular ovarian cycles were maintained in groups with a fertile male and up to five progeny. Ovarian cycles were controlled by i.m. injection of 0.75 μg of the prostaglandin $F_{2\alpha}$ analog cloprostenol (Estrumate; Mobay Corp., Shawnee, KS) during the middle to late luteal phase [7]. Blood samples (0.2 ml) were collected in heparinized syringes on Day 0 (immediately before cloprostenol injection), and on Days 3, 7, 9, 11, and 13. Plasma progesterone concentrations were determined by ELISA [10]. The day of ovulation was taken as the day preceding a plasma progesterone concentration of 10 ng/ml or more [8]. Eight days after ovulation, marmosets were lightly anesthetized by the i.m. injection of alphaxalone and alphadolone (Saffan; Glaxovet, Ltd., Uxbridge, UK), and blastocysts were recovered by a nonsurgical uterine flush procedure [9].

Blastocysts were incubated in 0.5% pronase-Dulbecco's

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Modified Eagle Medium (DMEM) while observed under a binocular microscope. Immediately after zona pellucida dissolution, the blastocysts were washed through two changes of DMEM. To remove the trophectoderm by immunosurgery [11], the blastocysts were exposed to a 1:50 dilution of rabbit anti-marmoset spleen cell antiserum in DMEM for 30 min, washed three times in DMEM, and then incubated in a 1:10 dilution of guinea pig complement (Gibco Labs., Grand Island, NY) for 30 min. After two further washes in DMEM, lysed trophectoderm cells were removed from the intact inner cell mass (ICM) by gentle pipetting, and the ICM was plated on inactivated (3500 rads gamma irradiation) mouse embryonic fibroblasts. Unless otherwise noted, culture medium consisted of 80% Dulbecco's Modified Eagle's Medium (DMEM; no pyruvate, high-glucose formulation, Gibco BRL), with 20% fetal bovine serum (Hyclone Labs., Logan, UT), 0.1 mM β-mercaptoethanol (Sigma Chemical Company, St. Louis, MO), and 1% nonessential amino acid stock (Gibco BRL) [12]. After 7–10 days, ICM-derived masses were removed from endoderm outgrowths, exposed to 0.05% Trypsin-EDTA (Gibco BRL) for 3–5 min, and gently dissociated by gentle pipetting through a micropipette. Dissociated cells were replated on embryonic feeder layers in fresh medium and observed for colony formation. Colonies composed of closely packed cells with high nuclear/cytoplasmic ratios were individually selected, split again, and cultured in the same DMEM-supplemented medium. Early passage cells were frozen and stored in liquid nitrogen [12]. Cell lines were karyotyped with a standard G-banding technique and compared to published karyotypes for the common marmoset [13].

Cell Surface Markers

Cell lines Cj11 and Cj62 cultured on a layer of mouse embryonic fibroblasts were used to examine the expression of cell surface markers. Alkaline phosphatase was detected histochemically after fixation of cells with 100% ethanol, with "Vector Blue" (Vector Labs., Burlingame, CA) used as a substrate, as described by the manufacturer. The SSEA-1, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81 antigens were detected by immunocytochemistry with specific primary monoclonal antibodies (gifts of Peter Andrews, University of Sheffield, Sheffield, UK) and were localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC system, Vector Labs.) [14–16]. NTERA2 cl.D1, a pluripotent human EC cell line (gift of Peter Andrews, University of Sheffield), was used as a negative control for SSEA-1, and as a positive control for SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81 [17, 18]. Mouse ES cells (ES-jt3) were used as a positive control for SSEA-1, and as a negative control for SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81.

Differentiation

Embryo-derived marmoset cells were plated at low density in the absence of fibroblasts on gelatin-treated four-well tissue culture plates (Nunc, Roskilde, Denmark) in the same medium used for initial cell line isolation, but with either 0 or 1000 U/ml of added human leukemia inhibitory factor (LIF; Gibco). The resulting differentiated cells were photographed 8 days after plating.

RNA was prepared by guanidine isothiocyanate-phenol/chloroform extraction [19] from cultures of Cj11 and Cj62 cells grown on embryonic fibroblasts and allowed to differentiate spontaneously for 2 wk after achieving confluence. The mRNAs for α-fetoprotein (αFP), the α- and ß-subunits of CG (CGα and CGβ), and GnRH were detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Primers for the αFP, CGα, and CGβ mRNAs were as previously reported [6]. Primers for GnRH were based on human sequences of the second and fourth exons (5′ primer = (5′) *gggtcgac*TCCAGCCAGCACTGGTCCTATGG; 3′ primer = (5′) *gggtcgac*CTGCCCAGTTTCCTCTTCAATCAG) and amplify a cDNA of 219 bp (lower-case italics indicate nucleotides added to facilitate subcloning) [20]. The identities of the αFP, CGα, CGβ, and GnRH cDNAs were verified by subcloning and sequencing (not shown). Homology of all marmoset cDNAs with the human sequences was > 90%.

To measure the response of CG secretion to a GnRH agonist, Cj62 cells were plated on two four-well plates (Nunc) and allowed to grow to confluence. At confluence, the medium of one plate was supplemented with the GnRH agonist D-Trp^6-Pro^9-NHEt [21] at 0.30 nM; a second control plate was left unsupplemented. Medium was changed every other day for 2 wk, and fresh agonist was prepared from frozen 100-strength stocks at each medium change. Medium from each individual well was assayed for LH/CG activity by a mouse Leydig cell bioassay [22].

For embryoid body formation, Cj62 cells were grown beyond confluence on fibroblast feeder layers and allowed to spontaneously differentiate for 4 wk. One embryoid body was sectioned at 1 μm, rinsed twice with 0.1 M cacodylate buffer, pH 7.0, fixed with Karnovsky's fixative, post-fixed with osmium, and embedded in epoxy. One-micrometer sections were stained with toluidine blue dye and examined under a light microscope; selected ultrathin sections were stained with lead citrate and uranyl acetate, and examined on a Phillips 410 transmission electron microscope.

RESULTS

Eight pluripotent cell lines were derived from marmoset blastocysts, two of which, Cj11 and Cj62, were cultured continuously for over 12 mo; the others were frozen at 3–8 mo of culture for later analysis. These cells had a high nucleus:cytoplasm ratio, prominent nucleoli, and a compact colony morphology (Fig. 1A) similar to, but distinguishable from, that of human EC cells. Cj11 and Cj62 cells expressed a series of cell surface markers (alkaline phosphatase, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81) that characterize undifferentiated human EC cells and rhesus ES cells (Fig. 2) [6, 16, 18, 23]. Immunostaining for SSEA-3 and TRA-1–81 was weaker than that for SSEA-4 and TRA-1–60, and the intensity varied both between and within colonies. Each of the marmoset cell lines had a modal chromosome number of 46; seven were XX and one was XY (Table 1).

When each of the eight pluripotent marmoset lines was

TABLE 1. Karyotypes of pluripotent marmoset cell lines.

Cell line	Chromosome number	Sex
Cj11.2	46*	XX
Cj25.1	46*	XX
Cj28	46	XY
Cj33	46*	XX
Cj35	46*	XX
Cj36	46*	XX
Cj39	46	XX
Cj62	46*	XX

* Some rare cells with abnormal karyotypes were observed, including fused chromosomes, marker chromosomes, and extra chromosomes.

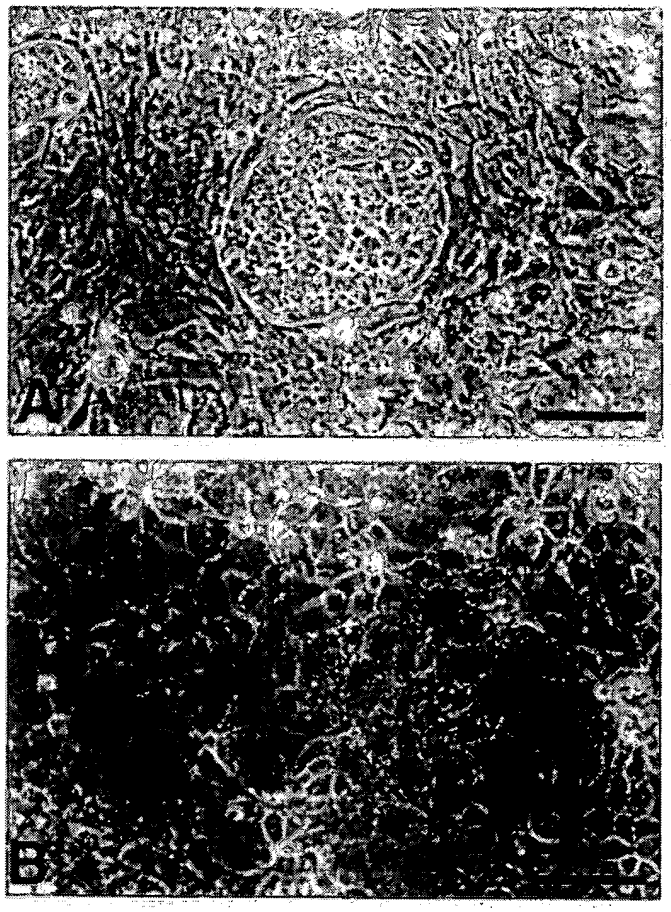

FIG. 1. Colony morphology and in vitro differentiation of cell line Cj62. A) Undifferentiated Cj62 cells on a background of embryonic fibroblasts. Note the distinct colony border, high nucleus:cytoplasm ratio, and prominent nucleoli (bar = 100 μM). B) Differentiated cells 8 days after Cj62 cells were plated on gelatin-treated tissue culture plastic, with 10^3 U/ml added human LIF (bar = 100 μM).

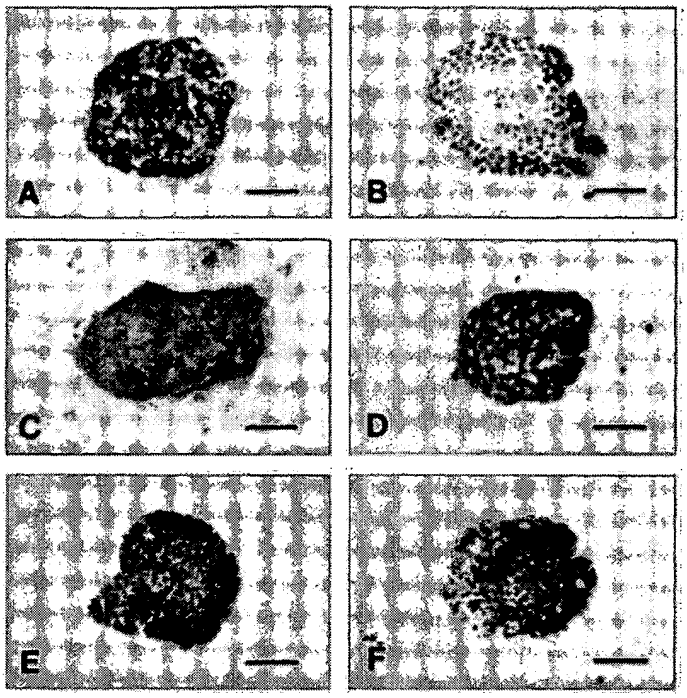

FIG. 2. Expression of cell surface markers by undifferentiated Cj62 cells (bar = 100 μM). A) Alkaline phosphatase (Vector Blue substrate). Because no counterstain was used, the fibroblast feeder layer is not visible. B) SSEA-1. C) SSEA-3. D) SSEA-4. E) TRA-1-60. F) TRA-1-81. For panels B-F, detection was with horseradish peroxidase/diaminobenzadine, and positive cells are brown. Counterstaining was with hematoxylin. Although consistently positive, SSEA-3 and TRA-1-81 staining of Cj62 cells was weaker than SSEA-4 and TRA-1-61 staining, and cell-staining intensity varied within and between colonies.

removed from fibroblast feeders, they differentiated into cells of several distinct morphologies, even in the presence of human LIF (Fig. 1B). The cells also differentiated when allowed to grow beyond confluence on fibroblast feeder layers. Among the differentiated cells derived from Cj11 and Cj62, trophectoderm was indicated by the expression of the CGα and CGβ mRNAs detected by RT-PCR (Fig. 3), and by the secretion of bioactive CG into the culture medium (Fig. 4). Differentiated cells also expressed mRNA for GnRH (Fig. 3), and the secretion of bioactive CG increased substantially when differentiating cells were exposed to GnRH agonist (Fig. 4). Endoderm differentiation (probable extra-embryonic endoderm) was indicated by the presence of αFP mRNA, detected by RT-PCR (Fig. 3).

When each of the eight pluripotent marmoset cell lines was grown at high density, over a period of 1–2 wk epithelial cells differentiated and covered the culture dish; the remaining groups of undifferentiated cells contracted into compact balls and then formed embryoid bodies. Over 3–4 wk, some of the embryoid bodies formed a bilaterally symmetric pyriform embryonic disc, an amnion, a yolk sac, and a mesoblast outgrowth attaching the caudal pole of the amnion to the culture dish. Histological and ultrastructural examination of one of these embryoid bodies (formed from a cell line, Cj62, that had been passaged continuously for 6 mo) revealed a close resemblance to an early primitive

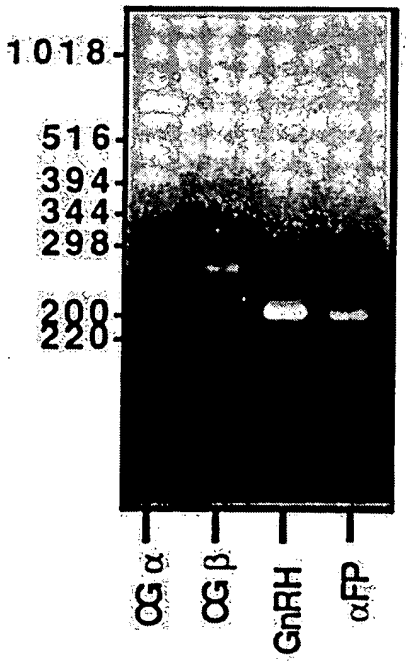

FIG. 3. RT-PCR amplification of mRNAs for CGα, CGβ, GnRH, and αFP from total RNA from pluripotent marmoset cells allowed to differentiate in vitro. Identities of all cDNAs were confirmed by subcloning and sequencing. Negative controls, which contained no template DNA, produced no bands (not shown).

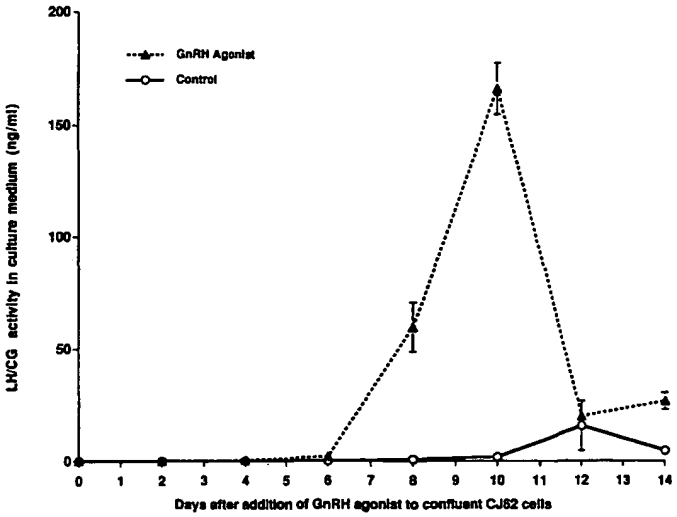

FIG. 4. GnRH agonist D-Trp^6-Pro^9-NHEt responsiveness of CG secretion in differentiating Cj62 cells. LH/CG bioactivity was measured by Leydig cell bioassay in culture medium conditioned by differentiating Cj62 cells. GnRH agonist was added to undifferentiated Cj62 cells at confluence (Day 0); medium was changed every 2 days and supplemented with fresh agonist. Bars represent SEM.

streak-stage embryo (Fig. 5). The embryonic disc was composed of a polarized, columnar epithelial epiblast (primitive ectoderm) layer separated from a hypoblast (primitive endoderm) layer. Electron microscopy of the epiblast revealed apical junctional complexes, apical microvilli, subapical intermediate filaments, and a basement membrane separating the epiblast from underlying endoderm—all features of the normal embryonic disc. In the caudal third of the embryonic disc, there was a midline groove, disruption of the basement membrane, and mixing of epiblast cells with underlying endoderm cells (early primitive streak; Fig. 5). An amnion was composed of an inner squamous (ectoderm) layer continuous with the epiblast, and an outer mesoderm layer.

DISCUSSION

Our criteria for ES cells are as follows: derivation from the preimplantation embryo, immortality, a normal karyotype, and the maintained ability to differentiate to derivatives of all three embryonic germ layers. Contribution to the germ line in chimeras is also a property of some mouse ES cell lines, but originally the term was introduced to distinguish the origin of pluripotent mouse cell lines derived from preimplantation embryos (ES cells) from those derived from teratocarcinomas (EC cells) [3]. Although mouse ES and EC cells are very similar, ES cells generally have a greater developmental potential, a difference thought to be related to the selective pressures of the teratocarcinoma environment that are avoided by the in vitro derivation of ES cells [3].

Several characteristics of the pluripotent marmoset cell lines we have isolated make them strong candidates for ES cells. First, the pluripotent marmoset cells continue to proliferate rapidly for at least 18 mo in continuous culture, and at least some maintain a normal karyotype. Although spontaneously immortal cell lines have been derived from primary cultures of mouse cells, this occurs rarely, if ever,

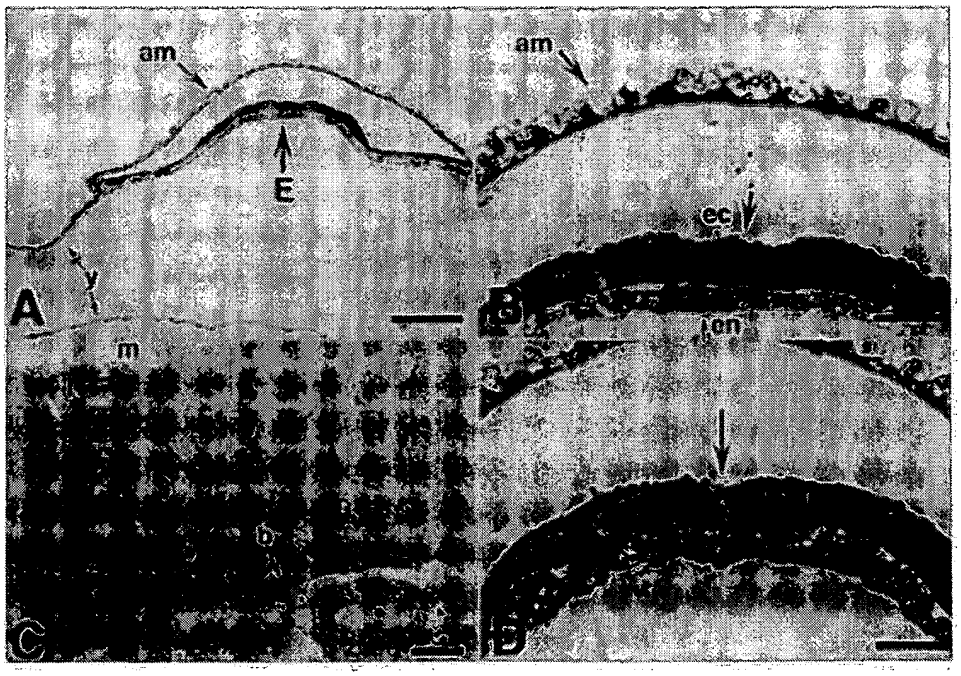

FIG. 5. Embryoid body formed from cell line Cj62 after 6 mo of undifferentiated culture. Cj62 cells were grown to confluence and then were allowed to spontaneously differentiate for 4 wk. **A)** Structures with morphological characteristic of yolk sac (y), amnion (am), and bilayered embryonic disc (E). The yolk sac was spherical, but collapsed during embedding, and the portion of the yolk sac to the right of the photograph was trimmed so the block would fit the diamond knife for sectioning. The embryonic disc was pyriform-shaped with a central groove in its caudal (narrow) aspect, and was connected to the tissue culture plate at its caudal pole by a stalk of mesenchymal cells. (Bar = 200 μM, toluidine blue stain). **B)** Section in cranial 1/3 of embryonic disc. Note that the primitive ectoderm (ec) forms a distinct cell layer from the underlying primitive endoderm (en), with no mixing of cell layers. Note also that the amnion (am) is composed of two distinct layers; the inner layer is continuous with the primitive ectoderm at the margins. (Bar = 50 μM, toluidine blue stain). **C)** Electron micrograph of embryonic disc. Apical microvilli (m) and apical junctional complexes (j) are present in the ectoderm layer, and the basement membrane (b) separates the ectoderm from the underlying endoderm. (Bar = 5 μM, lead citrate and uranyl acetate). **D)** Section in caudal 1/3 of embryonic disc. Note the central groove (arrow) and the mixing of primitive ectoderm and endoderm. This is the approximate level of early primitive streak formation in the normal primate embryo. (Bar = 50 μM, toluidine blue stain).

from primary cultures of somatic cells of primates, which consistently undergo crisis after a characteristic number of cell divisions [24]. Our success in isolating multiple immortal cell lines from both rhesus monkey and marmoset ICMs suggests that, unlike adult somatic cells, the undifferentiated, totipotent cells of the early embryo are immortal; that is, they are capable of unlimited proliferation. A second characteristic of the pluripotent marmoset cells is the expression of SSEA-3, SSEA-4, TRA-1–60, TRA-1–81, and alkaline phosphatase—a combination of cell surface markers previously described only for rhesus monkey ES cells and human EC cells [6, 14–16, 18]. The differentiation of human EC cells results in the loss of SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81 expression, and the earliest lineages to differentiate from the ICM in the human embryo, extra-embryonic endoderm and trophoblast, lack this combination of markers [18]. A third important characteristic of the pluripotent marmoset cells is the potential to differentiate to both endoderm and trophoblast, as the last cells in the mammalian embryo capable of contributing progeny to both these lineages are the totipotent early ICM cells [25]. And finally, the pluripotent marmoset cells differentiate to embryoid bodies with a remarkable resemblance to postimplantation early primitive streak-stage embryos [26].

Pluripotent marmoset cells offer an important new in vitro model for studying the differentiation and function of tissues that differ significantly between mice and primates. For example, the structure and the function of the trophoblast, which forms the outer layer of the placenta, differs dramatically between primates and rodents. Trophoblast secretion of CG in primates, including humans, is central to the maternal recognition of pregnancy. The mouse placenta does not express a CG, and mouse ES cells fail to differentiate to trophoblast or do so infrequently [27]. If the primate corpus luteum is exposed to CG, progesterone secretion is continued and pregnancy is maintained; in the absence of CG, the corpus luteum regresses, progesterone secretion declines, and a new ovarian cycle is initiated. GnRH is expressed in the placenta and has been proposed to have a local regulatory role in CG secretion [28]. The increase in CG secretion we observed in differentiating pluripotent marmoset cells in the presence of GnRH agonist supports a role in CG expression. Further, the dramatic effects observed suggest that GnRH may not only act *on* differentiated trophoblasts but might also be directly involved in the differentiation *of* trophoblasts. GnRH has been shown to be expressed and have biological effects in extrapituitary tissues other than the placenta [29–31], and this may point to a wider role for this regulatory peptide in differentiation and development. Because these pluripotent marmoset cells can be grown indefinitely, prior to differentiation it will be possible to use homologous recombination to modify trophoblast-specific genes, such as CG, GnRH, or their receptors, to help elucidate their function and regulation during and after differentiation.

The pluripotent marmoset cells initiate the formation of all three germ layers in embryoid bodies. If culture conditions can be established that allow efficient, synchronous development of organized embryoid bodies, then it will be possible to use these pluripotent marmoset cells to genetically dissect in vitro the mechanisms controlling early primitive streak formation in primates. We are not aware of primitive streak formation occurring in mouse embryoid bodies, which exhibit a more disorganized development [32]. With our present culture conditions, however, embryoid body formation is asynchronous, and many embryoid bodies develop into simple multilayered vesicular structures without the well-organized structure represented in Figure 5. To date, we have not observed development of embryoid bodies beyond the initiation of primitive streak formation, which is also the approximate stage where intact marmoset embryos degenerate in our culture conditions. To rigorously test the developmental potential of these pluripotent marmoset cells, it will be necessary to provide them with a normal embryonic environment, in chimeras with intact embryos.

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Exhibit D.

While researchers in many countries engage in political battles over human embryonic stem cells, Israeli scientists have moved to the vanguard of this scorching field

In the Mideast, Pushing Back The Stem Cell Frontier

JERUSALEM AND HAIFA—The other passengers on the flight from Singapore to Australia never suspected that a small flask of reddish liquid tucked in Benjamin Reubinoff's shirt pocket contained what is now one of the hottest commodities in biomedical research. It was September 1998, and the fertility specialist was carrying one of the first preparations of human embryonic stem (ES) cells.

Reubinoff was a long way from his home in Israel. In January 1998, the gynecologist at Hebrew University's Hadassah Medical Center in Jerusalem had come to Australia to spend a 1-year sabbatical with Alan Trounson and his colleagues at Monash University in Melbourne. Reubinoff had joined a high-risk project: Trounson's team had struggled for years to derive ES cells from human embryos. "We could get them formed but couldn't keep them maintained" for long, Trounson recalls, so the effort had stalled. Reubinoff knew nothing about the top-secret work when he first approached Trounson, a well-known embryologist and in vitro fertilization (IVF) expert, about a place in his lab. When Trounson filled him in, Reubinoff jumped at the opportunity.

At the forefront. In 1998, Benjamin Reubinoff helped energize a pioneering stem cell project in Australia before coming home to the Hadassah Medical Center to establish his own research effort.

Reubinoff's enthusiasm and grit at the lab bench—along with that of developmental biologist Martin Pera of Oxford University—energized the project. Because the state of Victoria outlaws research using human embryos, Trounson was collaborating with Ariff Bongso at the National University of Singapore, where no such prohibitions existed. So in August 1998, Trounson dispatched Reubinoff to Singapore for another attempt at creating a stable line of human ES cells. Within a few weeks the team succeeded, deriving the cell line that Reubinoff then kept warm in his shirt pocket on the flight to Australia.

Back at Monash, Reubinoff logged 15-hour days for months to concoct a recipe that would keep the vexing cells dividing but not maturing. "His persistence in the face of frustration really made the project work," says Trounson. Reubinoff's sabbatical extended into a 2-year stay and a Ph.D. He also helped put Israel on the stem cell map.

With their magical potential to transform into any cell type in the body, human ES cells have kindled hopes for new treatments for the millions of sufferers of Parkinson's disease, Alzheimer's, and other killers that share a hallmark feature: cell death. But because ES cells are culled from early embryos that are destroyed in the process, they are at the center of heated debates over research ethics and the sanctity of life.

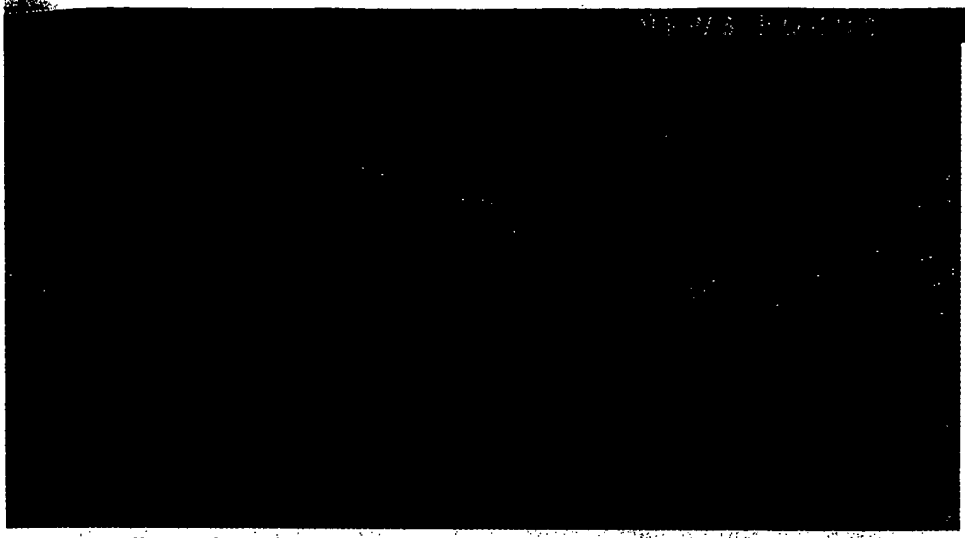
Indeed, in many countries, biologists have spent more time lobbying politicians and courting public opinion than they have in their labs learning about the cells. But that's not the case in Israel. Thanks to liberal regulations governing embryo research and broad public support, scientists here have been at the forefront of ES cell research. Reubinoff and gynecologist Joseph Itskovitz-Eldor of the Rambam Medical Center at the Technion in Haifa were key players in the landmark isolation of stem cells from human embryos in 1998. And of the first 12 publications on human ES cells 10 included Israeli authors. "There's less of a pall over the work in Israel," says stem cell expert George Daley of the Massachusetts Institute of Technology in Cambridge, who collaborates with Itskovitz-Eldor.

Because of their head start, Israeli scientists have helped set the pace for the rest of the world. Researchers here, with U.S. collaborators, were the first to publish detailed descriptions of the differentiation of human ES cells in culture in October 2000, and they were the first to report the genetic modification of the cells months later. The Israeli team "are very important players" in doing the fundamental work of figuring out how the human cells work, says Ron McKay of the National Institute of Neurological Disorders and Stroke in Bethesda, Maryland, a specialist on mouse ES cells.

Far-flung connections

When James Thomson of the University of Wisconsin, Madison, and his colleagues first announced the isolation of stem cells from human embryos in November 1998, the news took the scientific community by storm. Although several groups had been racing toward this goal, they had mostly kept their progress under wraps—to prevent tipping off competitors and to avoid the tumultuous public attention that has buffeted the young field ever since.

Although the initial successes happened about as far from Israel as one can get—in Wisconsin and in Melbourne—both teams had Israeli collaborators. Thomson was working with Itskovitz-Eldor, who in 1998 had sent him more than a dozen frozen embryos donated by Israeli couples in IVF clinics. One of Itskovitz-Eldor's graduate students, Michal Amit, carried the frozen embryos to Thomson's lab and assisted in the project. Four of the five cell lines the team first described (*Science*, 6 November 1998, p. 1145) came from Israeli embryos. Just before publication, Itskovitz-Eldor carried cells from all five lines back to Israel.



Stem cell central. Researchers from six countries (red arrows) have shuttled to Israel to study the fine art of stem cell science. So far Israel has shared its human ES cell lines with scientists in one country, the United States (blue arrow).

Itskovitz-Eldor and Reubinoff credit advances in IVF techniques for making the derivations possible. Scientists could not simply follow the recipe used to create most mouse ES cell lines. These are derived from blastocysts, which develop about a week after fertilization when the embryo forms a shell around a cluster of cells called the inner cell mass. The blastocysts were flushed from pregnant mice to get the inner cell mass, which gives rise to ES cells. As that method would never be morally acceptable in pregnant women, researchers knew they would have to find a way to derive ES cells from test tube embryos. In the mid-1990s, says Reubinoff, "there was a big question whether it could be done."

The problem was that researchers did not know how long human embryos could last outside the body. Until the mid-1990s, the normal routine at IVF clinics was to transplant embryos into a patient about 3 days after fertilization, before the inner cell mass develops. However, hypothesizing that embryos that survive to develop healthy blastocysts would be more likely to establish a successful pregnancy, IVF researchers in the United States and Australia developed methods to keep embryos alive longer in culture. Given the importance of IVF expertise and connections to the research, it isn't surprising that Israeli researchers were involved, says Reubinoff. "According to Jewish tradition, to procreate is very important," he says. "There is a lot of support for infertility treatments and a very large number of IVF clinics in Israel."

Fundamental firsts

When Thomson's team introduced the world to human ES cells, developmental geneticist Nissim Benvenisty of Hebrew University in Jerusalem leapt to take advantage of the breakthrough. As one of a handful of researchers who had studied how mouse ES cells transform into mature cells, Benvenisty had long been captivated by the potential of using human ES cells to probe early development and perhaps to treat diseases. "I had been waiting for years for someone to isolate human ES cells," he says. The day he read about the Wisconsin findings, he phoned co-author Itskovitz-Eldor, just 2 hours away in Haifa, to discuss the work. The conversation went so well that Itskovitz-Eldor drove to Jerusalem later that week to hash out a collaboration.

Their first project was to test whether human ES cells, like those from mice, can form clusters of differentiating cells called embryoid bodies. Initial studies by Thomson's group suggested they didn't. But by learning how to grow the cells suspended in liquid rather than flat on a dish, the labs found that they could. "[Benvenisty] is excellent at taking what has been done in the mouse cells and translating it to human cells," says developmental geneticist Austin Smith of the University of Edinburgh, U.K.

Benvenisty and company scored several other firsts. In October 2000 the team, along with cell biologist Doug Melton of Harvard University, published the first paper on how growth factors, such as bone morphogenic protein 4 and fibroblast growth factor, prompt human ES cells to mature into different cell types. They struck again last spring with the first report on the stable genetic modification of human ES cells. In that work, they inserted into stem cells a gene for green fluorescent protein, which glows in immature cells and shuts off as they begin to differentiate. The cell line should prove a boon to research, as it enables researchers to easily sort immature ES cells from those that have begun to transform.

Benvenisty's group is also collaborating with Melton to tackle a mountain on the stem cell landscape: targeting genetic changes to a specific gene or spot in the genome. This would allow researchers to knock out or modify specific genes. Success would bring them closer to the Benvenisty lab's ultimate goal: creating human ES cells that are not attacked by the immune system. Cells in the body display proteins called HLA antigens that help the immune system tell friend from foe. Like organ transplants, ES cells infused in a patient would trigger a potentially fatal reaction unless the immune system were suppressed, but suppression triggers a host of side effects that could doom a potential treatment. One way to avoid the problem, says Benvenisty, might be to knock out or shut off the HLA genes. Such modified cells could be a universal donor, like type-O blood, accepted by all patients' immune systems.

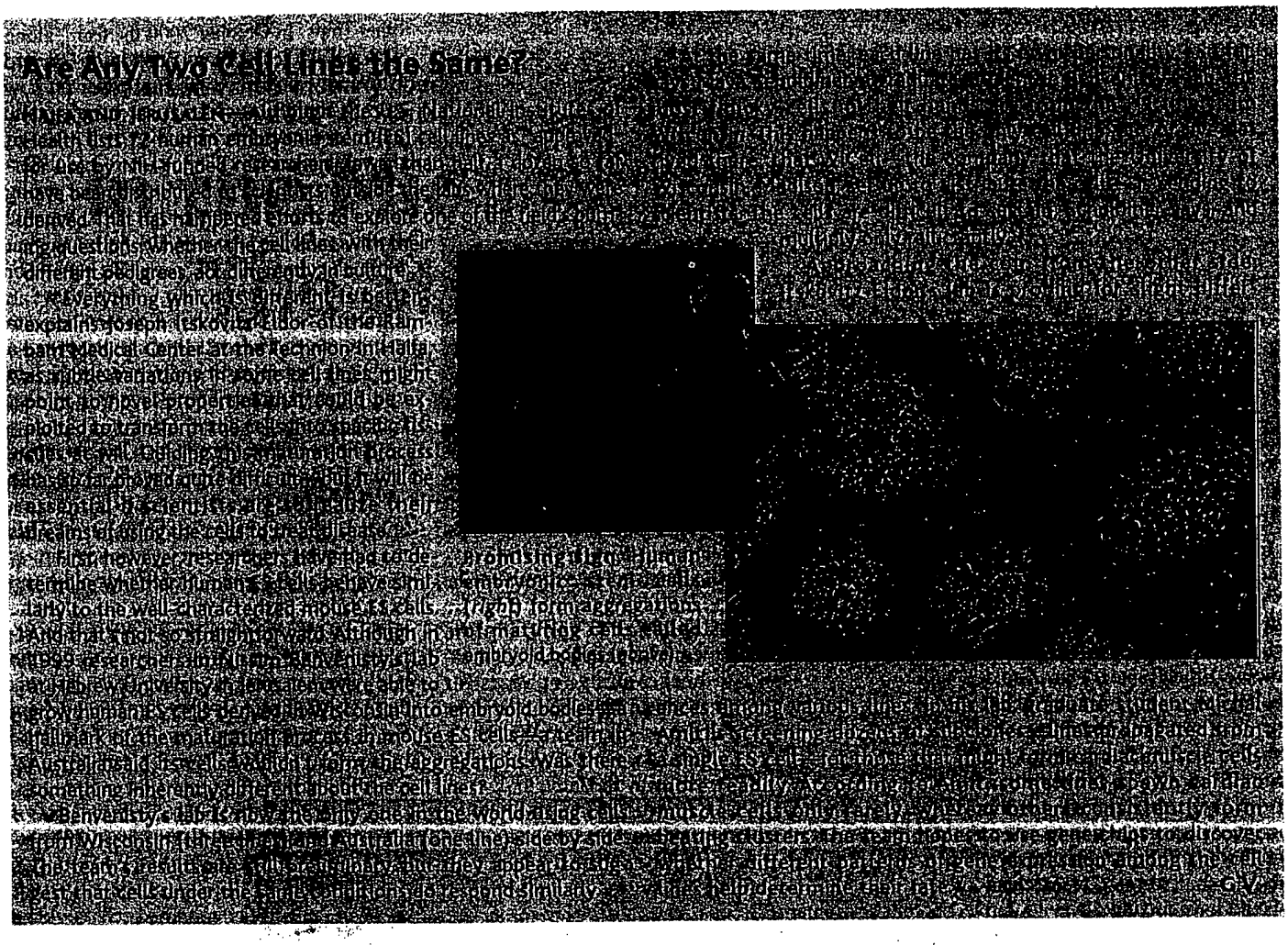
A northern leading light. Joseph Itskovitz-Eldor's lab in Haifa has managed to coax ES cell lines to grow on human "feeder" cells.

CREDIT: (BOTTOM) COURTESY OF J. ITSKOVITZ-ELDOR

Mouse-free ES cells

A few hours north of Jerusalem, in a 13th-floor lab with a sweeping view of the sun lovers on Haifa beach and the Mediterranean's turquoise waters, Itskovitz-Eldor's team has notched its own set of firsts. The group has recently managed to overcome one practical hurdle standing in the way of using human ES cells as therapy. To keep cells undifferentiated, scientists grow them on a "feeder layer" of embryonic mouse cells, which generates an as-yet-unknown cocktail of proteins that signal cells to remain immature. That means all existing human ES cell lines have been exposed to mouse cells—and possibly to unknown pathogens. Itskovitz-Eldor's group has figured out how to remove mice from the picture by growing ES cells on feeder cells derived from human fetal tissue.

Itskovitz-Eldor and Amit also continue to create new cell lines. They are working on a new method, in which embryos are allowed to develop several days beyond the blastocyst



stage. The researchers hope their latest cell lines might grow in culture more easily or develop into target tissues more readily. Although Itskovitz-Eldor is reluctant to discuss the issue, the new technique might also fall outside the broad patents owned by the University of Wisconsin that cover cell-line derivation using Thomson's method.

A few floors below Itskovitz-Eldor and his colleagues in Haifa, Karl Skorecki's lab in the Rappaport Institute at the Technion is studying lines of ES cells that have been tweaked genetically to churn out loads of telomerase, a protein that adds "caps" to the ends of chromosomes to protect them from degradation after multiple divisions. The team has shown that the enzyme, which is active in undifferentiated ES cells, normally shuts off as cells begin to differentiate. The hope is that differentiated cells in which telomerase stays active undergo more divisions, enabling researchers to grow larger batches of a tissue—a boon for the development of potential therapies.

Masters of translation. Getting human ES cells to perform like the well-studied mouse cell lines is what Nissim Benvenisty (far right) and his team at Hebrew University do best.

CREDIT: COURTESY OF N. BENVENISTY

Four musketeers

In a field known for its secrecy and competition, the Israeli teams stand out in another way: The four groups have linked up on a grant proposal to the Israeli Ministry of Science for up to 2 million shekels ($430,000). Their goal is to study how human ES cells develop into four key tissues: blood, pancreas, neurons, and liver. They hope that banding together on a broad proposal will convince the ministry to give them a relatively large chunk of Israel's limited medical research funding. "There is a lot of technology developed within the groups here," says Reubinoff. "If we can join forces, we can move the field more quickly forward."

And as more researchers worldwide gain access to the cells, the Israeli labs are becoming ever more popular, with scientists from half a dozen countries (see map, p. 1819) making pilgrimages to Jerusalem or Haifa to learn from the masters. "For scientists to make this technology applicable one day to patients requires collaboration with the whole world," Benvenisty says. And with stem cell advances pouring out of Israel, the steady flow of visitors seeking knowledge is unlikely to abate anytime soon.

–GRETCHEN VOGEL